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Zinc Stabilizes the SecB Binding Site of SecA[†]

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ABSTRACT: The molecular chaperone SecB targets preproteins to SecA at the translocation sites in the cytoplasmic membrane of *Escherichia coli*. SecA recognizes SecB via its carboxyl-terminal 22 aminoacyl residues, a highly conserved domain that contains 3 cysteines and 1 histidine residue that could potentially be involved in the coordination of a metal ion. Treatment of SecA with a zinc chelator resulted in a loss of the stimulatory effect of SecB on the SecA translocation ATPase activity, while the activity could be restored by the addition of ZnCl₂. Interaction of SecB with the SecB binding domain of SecA is disrupted by chelators of divalent cations, and could be restored by the addition of Cu²⁺ or Zn²⁺. Atomic absorption and electrospray mass spectrometry revealed the presence of one zinc atom per monomeric carboxyl terminus of SecA. It is concluded that the SecB binding domain of SecA is stabilized by a zinc ion that promotes the functional binding of SecB to SecA.

SecB is a molecular chaperone with a dedicated function in the translocation of preproteins across the cytoplasmic membrane of *Escherichia coli* (1, 2). SecB associates with the mature region of nascent secretory proteins while they emerge from the ribosome (3–5). Due to this association, SecB prevents the stable folding of preproteins and preserves a conformation that is competent for translocation (2, 6). Subsequently, SecB targets the preproteins to the membrane-bound SecA subunit of the translocase, the membrane protein complex that selectively transports presecretory proteins across the cytoplasmic membrane (7–9). SecA serves both as a receptor for preproteins and SecB and as an ATP-driven molecular motor driving the stepwise translocation of the polypeptide chain across the membrane (10).

SecB binds with high affinity to the translocase-bound SecA (7, 8, 11). A strikingly short amino acid sequence corresponding to only the most distal 22 aminoacyl residues of SecA suffices for the authentic binding of SecB or the SecB/preprotein complex (8). A mutant of SecA lacking this region still supports preprotein translocation but is unable to bind SecB. Since the SecB–SecA interaction is needed to dissociate the mature preprotein domain from SecB, the SecA lacking the SecB binding site is unable to support translocation of SecB-associated preproteins (9). The SecB–SecA interaction is strengthened by the binding of the signal sequence to SecA (7), thereby facilitating efficient transfer of the preprotein from SecB to the translocase (9).

With the exception of *Streptomyces*, *Mycobacterium*, and *Mycoplasma* species, the carboxyl terminus of SecA is nearly identical for most of the bacterial species. The SecB binding site contains three conserved Cys residues and a nonconserved His residue which, when absent, is replaced by a fourth Cys residue. These amino acids are often involved in the coordination of metal ions, but so far there is no evidence for a critical role of metal binding in the SecA function. The role of these residues in the SecA protein is unknown, but serine substitutions of the conserved Cys residues compromise the SecA function (12). These mutants are no longer able to support SecB-dependent translocation of preproteins. Furthermore, the reducing environment of the cytosol makes it unlikely that disulfide bond formation is a critical structural feature of this domain. In this respect, oxidizing or reducing conditions do not affect the SecA–SecB interaction at the membrane (8). We now demonstrate that the SecB binding site of SecA contains a zinc atom that is coordinated by Cys residues. Since zinc is needed for the functional binding of SecB to SecA, it is concluded that SecA is a metalloprotein.

EXPERIMENTAL PROCEDURES

Biochemicals, Strains, and Growth Conditions. Inner membrane vesicles were isolated from *E. coli* strain SF100 (*F*[−] Δ *lacX74 galE galK thi rpsL (strA) ΔphoA(PvuII), ΔompT*) (13) harboring plasmid pET340 (14) that permits overexpression of the SecYEG complex. Cells were grown aerobically at 37 °C on LB broth supplemented with 50 μg of ampicillin/mL. Overexpression and purification of SecA (15), SecB (16), proOmpA (17), the *Schistosoma japonicum* glutathione-S-transferase (GST)¹ and GST218 (8), and SecYEG (18) were performed as described.

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¹ Abbreviations: ESI-MS, electrospray ionization mass spectrometry; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine; GST, glutathione-S-transferase; MIANS, 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid.

Atomic Adsorption. Atomic adsorption spectroscopy on samples of 500 μL containing 5.5 mg of protein (GST218 or GST) and 50 mM Tris-HCl, pH 7.6, was performed by the analytical center of Organic and Molecular Inorganic Chemistry (OMAC) of the University of Groningen.

Electrospray Ionization Mass Spectrometry. The SecA(878–901) fragment was obtained by thrombin treatment (500 units, Sigma Chemical Co.) of glutathione–Sephacryl 4B bound GST218 in 5 mM Tris-HCl, pH 7.3, at 20 °C. Incubation was performed overnight according to the manufacturer's recommendations. The cleaved peptide was collected by elution with 0.5 mM Tris-HCl, pH 7.3, and the sample was desalted with a HR 10/10 fast desalting (G-25, Pharmacia, Uppsala, Sweden) column, which was preequilibrated with 10 mM ammonium acetate buffer (pH 7.3). The fraction with the highest protein content of the eluted fractions (0.5 mL), as determined by 17.5% SDS–PAGE, was used for electrospray ionization mass spectrometry (ESI-MS) analysis. One volume of the SecA(878–901) fragment in 10 mM ammonium acetate was mixed with 1 volume of acetonitrile or with 1 volume of acetonitrile and 0.1% trifluoroacetic acid before ESI-MS analysis. ESI-MS spectra were recorded on a R 3010 quadrupole mass spectrometer (NORMAG, Argenteuil, France) equipped with a custom-built pneumatically assisted electrospray (ion spray) ion source. All data were acquired with a mass range from 800 to 1700 (0.1 amu scan step, 2 ms dwell time). The average molecular masses of the proteins were calculated from the m/z peaks in the charge distribution profiles of the multiple charged ions (19) after averaging of 21 scans. Spectral deconvolution was performed on the peaks over the mass range from 800 to 1700 using the computer program MacSpec (Sciex). All molecular masses quoted in this paper are average, chemical atomic masses.

MIANS Labeling. Cys labeling experiments were performed with the fluorescent probe MIANS. A suspension of 0.1–0.18 μM GST or GST218 in 50 mM Tris-HCl, pH 7.5 (1.5 mL final volume), was incubated with 8 μM MIANS at 25 °C, and the fluorescence was monitored using excitation and emission wavelengths of 328 and 416 nm, respectively. Fluorescence experiments were performed with an SLM-Aminco Bowman Series 2 fluorometer using slit widths of 4 nm.

Other Techniques. Binding of SecB to GST218 was performed as described (8). The translocation ATPase activity of SecA was assayed in the presence of SecYEG proteoliposomes as described (18). Protein determination was performed as described (20) using BSA as a standard.

RESULTS

Metal Chelators Inhibit the SecB-Stimulated Translocation ATPase Activity of SecA. The SecB binding site of SecA, which is located in the extreme carboxyl terminus (8), harbors three Cys and one His residue. Since such residues are often involved in the coordination of metal ions, we have employed a chelator that can be used to specifically remove heavy metals from proteins. The chelator N,N,N',N' -tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) binds zinc and other heavy metals with very high affinity; i.e., the K_d for Zn^{2+} is 10^{16} M^{-1} (21). On the other hand, magnesium and calcium are low-affinity ligands with K_d values of about 10^2

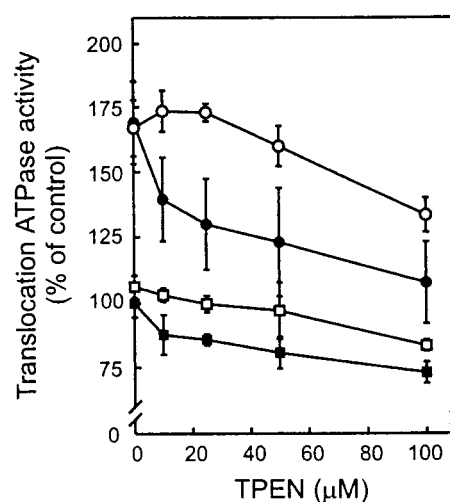


FIGURE 1: Zinc ions are needed for the functional interaction between SecA and SecB. The proOmpA-stimulated ATPase activity of SecA in the presence of SecYEG proteoliposomes (translocation ATPase) was measured in the presence of increasing concentration of TPEN (0–100 μM) in the absence of SecB and ZnCl_2 (■), in the presence of ZnCl_2 (□), in the presence of SecB (●), and in the presence of both SecB and ZnCl_2 (○). ZnCl_2 was added at 100 μM . The data were corrected for the background SecA ATPase activity in the absence of proOmpA. The standard deviation shown is the average of five independent experiments.

and 10^4 M^{-1} , respectively (21, 22). Therefore, at low concentrations, the chelator can be used to scavenge heavy metals from the solution without interfering with the binding of magnesium to SecA needed for the ATPase activity. In the absence of SecB, the translocation ATPase activity of SecA in the presence of SecYEG proteoliposomes was slightly inhibited by TPEN (Figure 1, ■). This phenomenon could be partially eliminated by the addition of 100 μM ZnCl_2 (□), although at high concentrations the SecA translocation ATPase was progressively inhibited (half-maximal inhibition at 750 μM). The presence of SecB results in a marked stimulation of the translocation ATPase activity of SecA (○, ●) (23). This stimulation could largely be prevented by treatment of the SecA with increasing concentrations of TPEN (○) while the activity could be restored by the addition of 100 μM ZnCl_2 (●). These additions had no effect on the SecA ATPase activity in the absence of proOmpA (data not shown). This result suggests that the metal chelator TPEN interferes with the functional interaction of SecB with SecA in a manner that can be restored by zinc ions.

Interaction of SecB with the Binding Domain of SecA Is Dependent on Divalent Cations. GST218, which is a fusion between the *Schistosoma japonicum* glutathione-S-transferase (GST) and the extreme carboxyl terminus of SecA, genuinely binds SecB (8). To directly access the effect of chelators and metals on the interaction between SecA and SecB, GST218 was treated with EDTA and subsequent dialysis against buffer without EDTA. This treatment resulted in a complete loss of the SecB binding activity (Figure 2A, compare lanes 1 and 2). Addition of extra EDTA did not further influence the residual binding (lane 3). SecB binding could be restored after addition of Zn^{2+} (lane 10) or Cu^{2+} (lane 9), and to a lesser extent by Co^{2+} (lane 5). Ni^{2+} , Ca^{2+} , Mn^{2+} , Fe^{2+} , Mg^{2+} , and Fe^{3+} (lanes 4, 6–8, 11, and 12) failed to restore SecB binding. Pretreatment of GST218 with TPEN

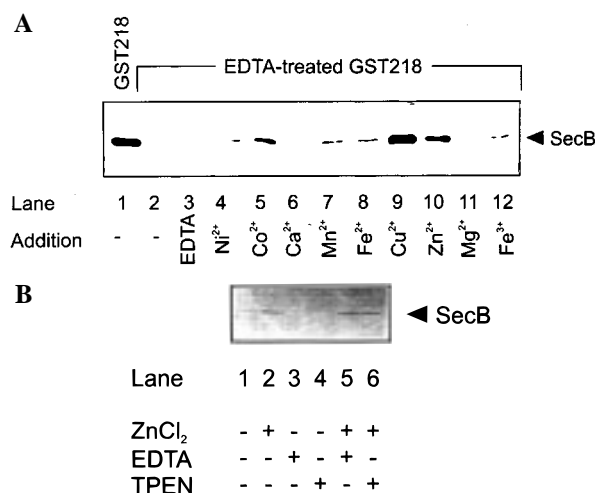


FIGURE 2: Effect of chelators and divalent cations on the binding of SecB to GST218. (A) SecB binding to GST218 is dependent on divalent cations. GST218 (lane 1) was washed with EDTA (lanes 2–12), and analyzed for its ability to interact with SecB in the presence of EDTA (2 mM) or various metal ions. All metal ions were added to a concentration of 100 μ M as chloride salts. Samples were analyzed by SDS–PAGE, blotted on PVDF, and immunostained with a polyclonal antibody against SecB. (B) The zinc chelator TPEN blocks SecB binding to GST218. Binding of SecB to GST218 was performed in the absence or presence of 5 mM ZnCl₂, 2 mM EDTA, or 2 mM TPEN. Samples were analyzed by SDS–PAGE, and the SecB protein was stained with Coomassie brilliant blue.

Table 1: Atomic Adsorption Spectroscopic Analysis of the Metal Content of GST and GST218

atom	content (ppm)	
	GST	GST218
Cu	<0.1	<0.1
Fe	<0.1	<0.1
Ni	<0.1	<0.1
Mn	<0.1	<0.1
Zn	<0.1	1640

also completely abolished its ability to interact with SecB, whereas the activity was restored after the addition of zinc (Figure 2B). The interaction of SecB with the binding domain of SecA therefore requires specific divalent metal ions.

The SecB Binding Domain of SecA Contains a Stoichiometrically Bound Zinc Ion. To determine the identity of the divalent cation present in the SecB binding domain of SecA, both GST and GST218 were examined for the presence of copper, iron, nickel, manganese, and zinc by atomic absorption spectroscopy. GST itself did not contain any of these metals, i.e., less than 0.1 ppm. Copper, iron, nickel, and manganese were also absent in GST218 (less than 0.1 ppm) (Table 1). However, the sample contained about 1 mol of Zn/mol of GST218.

The exact stoichiometry of zinc binding to the SecB binding domain of SecA was accessed by ESI-MS. The fragment corresponding to SecA(878–901) was purified to homogeneity by FPLC gel filtration after thrombin cleavage of the GST218. Both in the absence and in the presence of β -mercaptoethanol, the peptide was found to migrate as a single species on SDS–PAGE with an apparent molecular mass of about 2.5 kDa (data not shown), demonstrating that it is monomeric. The isolated peptide was subjected to ESI-MS. Reconstruction of the molecular masses of the Sec(878–

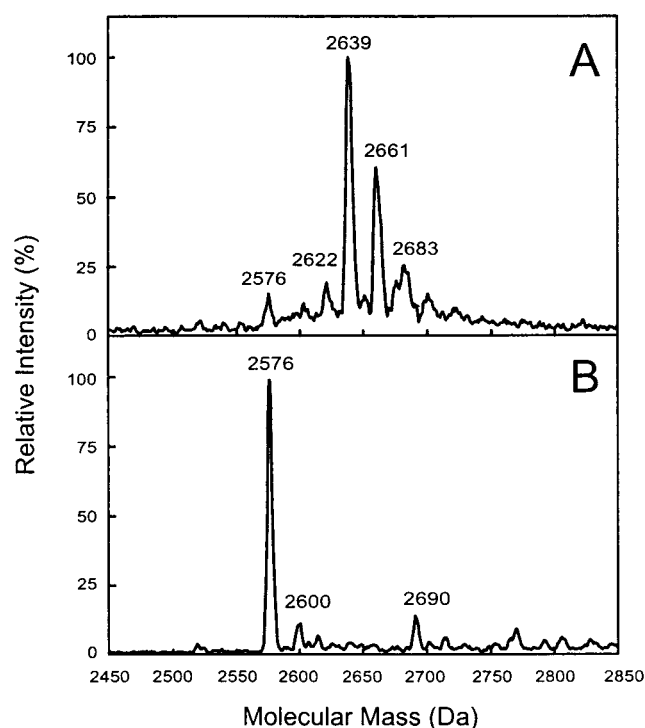


FIGURE 3: Electrospray ionization mass spectrometry of the SecB binding domain of SecA. (A) Spectrum of SecA(878–901) fragment in 10 mM ammonium acetate, pH 7.3. (B) Same as (A), but after the addition of 0.1% trifluoroacetic acid. The masses are indicated at the peaks.

901) fragment at pH 7.3 shows one main peak at 2639 Da, and four minor peaks at 2576, 2622, 2661, and 2683 Da (Figure 3A). The calculated average mass of the SecA(878–901) fragment is 2576.9 Da, which corresponds to the minor peak at 2576 Da. Binding of a divalent zinc ion to the SecA(878–901) fragment results in a displacement of two protons to maintain charge balance. Consequently, the net mass increase of SecA(878–901) is the mass of the Zn ion, which has an average isotopic mass of 65.4 Da, minus 2 Da, resulting in the 2639 Da peak in Figure 3A. The minor peaks at 2661 and 2683 Da correspond to the SecA(878–901) fragment plus one ligated Zn, with one and two sodium ions, respectively. The source of the sodium is unknown, but sodium is ubiquitous. Moreover, the presence of the bound sodium is often observed in the analysis by ESI-MS of divalent cation binding proteins without interfering with the specific protein–cation interaction (24). These data demonstrate that the SecB binding domain of SecA ligates zinc with a stoichiometry of 1:1.

Zinc Binding Involves the Carboxyl-Terminal Cysteines of SecA. Ligation of a zinc ion by Cys and/or His residues is known to be sensitive to pH changes (24, 25). Acidic conditions effectively result in the release of the ligated zinc ion (25). The reconstruction of the molecular masses of the SecA(878–901) fragment in the presence of 0.1% trifluoroacetic acid is shown in Figure 3B. Under these conditions, only one main peak at 2576 Da is evident that corresponds to the calculated mass of the SecA(878–901) fragment without zinc. The pH dependence of this interaction is consistent with an interaction of the zinc ions with the Cys and His residues.

The involvement of Cys residues in the coordination of the zinc ion was further analyzed by MIANS labeling. GST

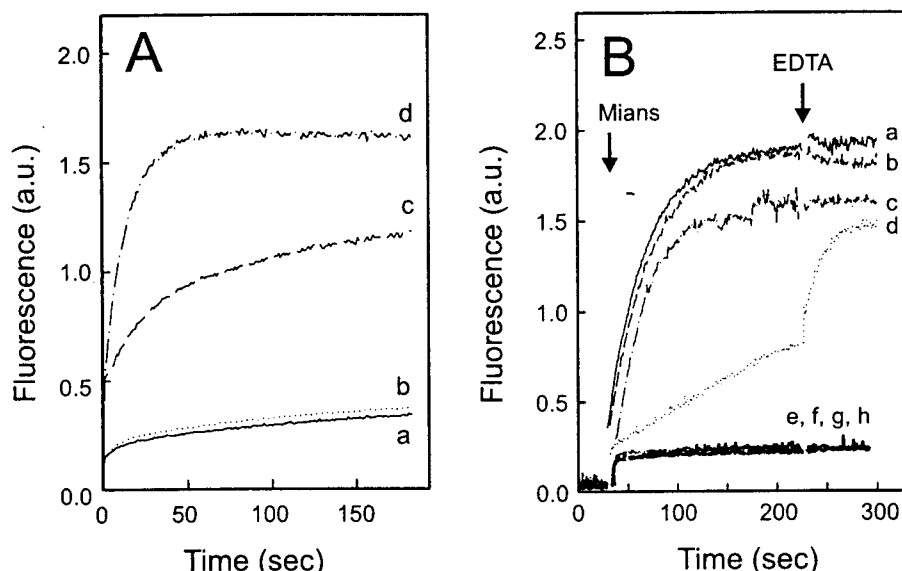


FIGURE 4: Cysteine residues are involved in zinc coordination. (A) Labeling of MIANS to GST in the absence (a) and presence (b) of 1 mM EDTA and labeling of MIANS to GST218 in the absence (c) and presence (d) of 1 mM EDTA. (B) Labeling of MIANS to GST218 (a–d) or GST (e–h) pretreated with EDTA in the absence (a, e) or presence of CaCl_2 (b, f), NiCl_2 (c, g), or ZnCl_2 (d, h). The arrow indicates the addition of extra EDTA.

contains three endogenous Cys residues that poorly react with MIANS (Figure 4A, trace a). Addition of EDTA hardly affected the labeling efficiency (trace b), implying that they are either present in the oxidized state or inaccessible for MIANS. GST218 contains three additional Cys residues in the SecB binding frame. MIANS readily labels one or more of these residues (trace c) in a manner that is markedly stimulated by EDTA (trace d) or TPEN (data not shown). When dialyzed against EDTA, the GST218 is immediately labeled with high efficiency by MIANS (Figure 4B, trace a), whereas the labeling of GST is negligible (trace e). The presence of zinc ions markedly reduced the rate of MIANS labeling of GST218 (trace d), but labeling was almost fully restored by the subsequent addition of EDTA. Calcium and nickel ions were unable to protect GST218 against MIANS labeling (traces b and c). These additions were without an effect on the labeling of GST (traces f–h). These results are consistent with coordination of the zinc atom by the Cys residues present in the SecB binding frame of SecA.

DISCUSSION

In this report, we demonstrate that the SecB binding site of SecA harbors a stoichiometrically bound zinc atom that is needed for the functional interaction between SecB and SecA. Chelators such as EDTA and TPEN destroy the binding of SecB to the SecB binding site of SecA, while the binding can be restored by zinc ions. The zinc chelator TPEN blocks the SecB-stimulated part of the SecA-translocation ATPase activity, and the activity can be restored by subsequent zinc addition. Previous studies with SecAN880, a truncate of SecA lacking the SecB binding site, have shown that this stimulation of the SecA-translocation ATPase activity by SecB strictly depends on the targeting function of SecB (8). Taken together, we conclude that SecA is a metalloprotein.

SecA not only exists, but also functions as a dimer (26), and both carboxyl termini of the SecA monomers are involved in the binding of the tetrameric SecB protein (8).

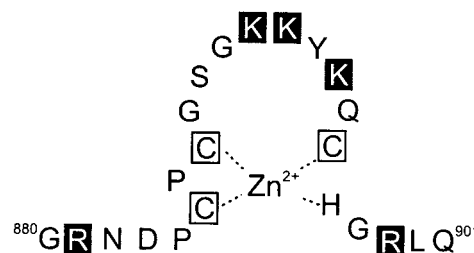


FIGURE 5: Hypothetical scheme of the coordination of a zinc atom by the SecB binding site of SecA. Positively charged amino acid residues are indicated in black, and the Cys residues are boxed.

Atomic absorption spectroscopy indicates that the zinc atom binds in a stoichiometric fashion to the carboxyl terminus of SecA. Thus, there will be two zinc atoms per SecA dimer. Although the carboxyl termini are in close proximity, there is evidence against a bridging function of the zinc atoms that would connect the two SecA monomers via their carboxyl termini. First, the isolated SecA(878–901) fragment migrates as a monomer on SDS–PAGE, both in the presence and in the absence of β -mercaptoethanol. Second, mass spectrometry of the peptide only provides evidence for a monomer that binds a single zinc atom. Removal of the zinc by a chelator will most likely unfold the SecB binding site since metal binding and protein folding in metal binding domains are tightly coupled (25). We therefore hypothesize that the zinc atom functions in stabilizing the carboxyl terminus of SecA as a high-affinity SecB binding site, without linking the two monomers. Essentially, the zinc atom stabilizes a tertiary fold with a net positive charge due to the presence of the conserved lysyl and arginyl residues (Figure 5). The predicted pI value of this domain is near pH 10. The zinc atom is likely to be liganded by deprotonated cysteine residues (27), although it is not certain if all three cysteines are anionic. Overall, the SecB binding region of SecA will contain an excess of positive charge. This strongly positively charged protein surface may electrostatically interact with the SecA binding site on SecB, which, at least partially, is composed of conserved negatively charged

residues (9, 28). These characteristics may also explain why the carboxyl terminus of SecA interacts with liposomes containing acidic phospholipids (12).

The cytosolic SecA binds SecB with poor affinity, i.e., K_d of 1–2 μ M (29), whereas the SecYEG-bound SecA is primed for the high-affinity interaction with SecB, i.e., K_d of about 30 nM (8). These two states of SecA may differ in their exposure of the SecB binding domain. For instance, binding of SecA to the SecYEG complex may elicit a conformational change whereupon the SecA exposes its carboxyl terminus for binding to SecB. This tightly membrane-associated and translocase-bound SecA exposes its carboxyl terminus to trypsin (15) and membrane-impermeable Cys-reactive probes (15, 30, 31) that are added from the periplasmic face of the membrane. SecB is released from the membrane (8) concomitantly with the membrane insertion of SecA (32), suggesting that the membrane surface and integrated forms of SecA differ in their exposure of the zinc-stabilized SecB binding site to the cytosol. These are also conditions that result in initiation of preprotein translocation, and the membrane insertion of the signal sequence and amino-terminal mature preprotein domain (33). A change in cytosolic to periplasmic exposure of the extreme carboxyl terminus of SecA may thus be part of the catalytic cycle of SecA at the membrane, and be coupled to the release of SecB from the translocase (8).

The conserved PCPCGSGKK[YF]KXC[HC]G motif (putative zinc coordinating ligands are underlined) corresponding to the SecB binding frame does not resemble any known zinc binding motif, and therefore defines a new type of binding fold. The high content of glycyl residues presumably provides this region with a high degree of flexibility to permit coordination of the zinc atom. Apart from a role of the Cys residues, it is not exactly known how these zinc atoms are coordinated. Most likely, the conserved His residue, which in some SecA proteins is replaced by a fourth Cys residue, is also involved in the ligation of zinc. In classical zinc fingers, the presence of four residues for coordinating the zinc atom suggests a structural zinc site (25, 34). However, in these structures the zinc binding ligands are never located directly next to each other in the primary sequence. Only in metallothioneins are adjacent Cys residues involved in binding the same metal atom (35). Thus, it seems that the SecB binding domain of SecA is a hybrid between the metal binding sites of metallothioneins and zinc-fingers. These and previous results (8) indicate that the carboxyl terminus of SecA suffices for authentic SecB binding. However, with the intact SecA, it cannot be excluded that in addition to the carboxyl terminus other parts of SecA contribute to the high-affinity interaction with SecB.

The mitochondrial Tim10 and Tim12, components of the inner membrane translocase, both harbor a zinc-finger domain with four Cys residues and bind equimolar amounts of zinc atoms (36). The interaction of Tim10 and Tim12 with the preprotein depends on the presence of zinc. Our current study with SecA shows that this phenomenon seems more general in protein translocation, although with SecA zinc is required for the functional binding of SecB rather than binding of the preprotein.

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